UNIVERSIDADE LUSÓFONA

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IMPLEMENTATION OF TWO INTRAOPERATIVE CYTOLOGICAL TECHNIQUES FOR EVALUATION OF CLEAN STATUS MARGINS IN FELINE MASTECTOMY

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Resumo

Os tumores mamários felinos são caracterizados por uma elevada frequência de disseminação metastática (Chocteau *et al.,* 2019) e são a terceira neoplasia mais comum na gata não esterilizada (Cassali *et al.*, 2020). A melhor abordagem para gatas com tumores mamários é a mastectomia bilateral com excisão de linfonodos, havendo a possibilidade de ser realizada em dois tempos cirúrgicos (Cassali *et al.*, 2020). A ressecção cirúrgica incompleta pode ocorrer em 45,6% dos pacientes felinos que realizaram mastectomias (Chocteau *et al.,* 2019) e, apesar do planeamento cirúrgico adequado, as técnicas de avaliação das margens intraoperatórias podem influenciar positivamente o tempo de vida do paciente.

O principal objetivo da cirurgia oncológica é conseguir uma ressecção microscópica livre de células tumorais, isto é, remover completamente o tumor do local cirúrgico (Bray *et al.*, 2023). Uma margem é definida como a distância entre o bordo do tecido excisado e qualquer tecido tumoral (Bakhshandeh *et al.*, 2006; Bray *et al.*, 2023).

A remoção completa de todo o tecido tumoral é um passo crucial para evitar recorrências na cirurgia oncológica (Bray *et al.*, 2023). Ao longo dos anos, e especialmente em medicina humana, surgiram várias técnicas de avaliação de margens intraoperatórias (Koopmansch *et al.*, 2021). Entre estas, destacam-se a citologia de impressão e a citologia de raspagem.

Cox *et al.*, 1991, foram os primeiros a implementar a impressão na avaliação intraoperatória de margens cirúrgicas em amostras de nodulectomia de mama em doentes humanos, com resultados encorajadores, uma vez que se registou uma precisão de 97,3%. D'Halluin *et al.*, 2009, efetuaram um estudo comparativo entre a citologia de impressão intra cirúrgica e a análise histopatológica pós-cirúrgica de 400 procedimentos de nodulectomia de mama em mulheres. Determinaram que, entre os dois métodos, havia uma correlação de 91,5%, com uma sensibilidade de 88,6% e uma especificidade de 92,2%, sendo o valor preditivo positivo e o valor preditivo negativo 73,6% e 97,0%, respetivamente.

Este novo método demorou, em média, apenas 10 minutos a ser executado (D'Halluin *et al.*, 2009). Esta técnica evitou uma segunda intervenção cirúrgica para correção de margens estreitas em 11,75% dos casos. Outros estudos realizados em pacientes humanos obtiveram resultados que não diferem destes, com sensibilidades e especificidades até 97% e 100%,

respetivamente, e valores preditivos positivos e valores preditivos negativos até 100% e 99%, respetivamente (Klimberg *et al.*, 1998; Bakhshandeh *et al.*, 2006).

A citologia de raspagem é uma modificação da citologia de impressão (Sharma *et al.*, 2021). Enquanto na impressão uma lâmina é suavemente pressionada ao longo da superfície da amostra excisada, na raspagem o leito da ferida deixado após a excisão é ligeiramente raspado, sendo o material recolhido posteriormente espalhado numa lâmina (Milovancev *et al.*, 2017; Sharma *et al.*, 2021). Vários estudos registaram uma elevada concordância entre esta técnica e a histopatologia (Kolte & Satarkar, 2010; Mahore *et al.*, 2015; Sharma *et al.*, 2021).

Kolte & Satarkar, 2010, avaliaram as capacidades de diagnóstico da raspagem numa miríade de tipos de tumores, tendo obtido uma precisão de 97,3% quando comparada com a histopatologia. De igual modo, tanto Mahore *et al.*, 2015, como Sharma *et al.*, 2021, testaram a raspagem no diagnóstico de tumores e obtiveram uma precisão de 93,49% e 96%, respetivamente.

A raspagem possui elevadas taxas de precisão, com preservação dos detalhes celulares sem danificar o tecido necessário para o diagnóstico histopatológico, sendo um método simples, barato e rápido (Shidham *et al.*, 2000; Kolte & Satarkar, 2010; Sharma *et al.*, 2021).

Para o presente estudo, foram recrutadas doze gatas com indicação de mastectomia para o tratamento cirúrgico de tumores mamários. O estudo foi efetuado durante um período de 16 meses. Todas as impressões foram preparadas a partir de cada espécime de mastectomia *ex vivo* após a excisão cirúrgica. Cada lâmina de microscópio foi suavemente pressionada contra cada margem de interesse da amostra. Todas as raspagens foram preparadas a partir das margens do campo cirúrgico com uma raspagem suave com uma lâmina de bisturi n.º 24 em cada superfície de interesse e espalhadas numa lâmina de microscópio. As amostras foram analisadas através de histopatologia *bread loaf*. Sessenta e nove margens elegíveis foram incluídas no estudo.

Relativamente à precisão de diagnóstico para a impressão e raspagem, não foram detetados verdadeiros positivos ou falsos negativos, tendo sido apenas detetados verdadeiros negativos e falsos positivos. A sensibilidade e o valor preditivo positivo para a impressão e raspagem foi de 0% para ambos, enquanto a especificidade, os valores preditivos negativos e a precisão para ambas as técnicas foram valores elevados.

Os resultados obtidos estão de acordo com estudos anteriores. As técnicas de citologia de impressão e citologia de raspagem foram fáceis de executar. São necessários mais estudos com um maior número de animais para compreender a relevância clínica destas técnicas no prognóstico dos tumores mamários felinos como técnicas complementares à histopatologia.

Palavras-chave: tumores mamários felinos, citologia de impressão, citologia de raspagem, histopatologia, sensibilidade, especificidade, valor preditivo positivo, valor preditivo negativo, precisão.

Abstract

Most mammary tumours in cats are malignant, with surgical removal to be the advocated treatment, achieved through bilateral radical mastectomy. In human medicine, several techniques were devised in order to save tissue in lumpectomies without compromising the patient's lives by leaving cancerous tissue behind. Cytological techniques stand out by being simple yet effective in the detection of residual tumoral tissue in the lumpectomy site.

This study was developed to test the feasibility of two cytological techniques, imprint cytology and scrape cytology, in the detection of margin status when compared to histopathology in feline mammary tumours.

Specificity and accuracy for imprint cytology and scrape cytology were, respectively, 98.6% and 95.7%. We concluded that both techniques are feasible in the assessment of surgical margins in cats undergoing radical mastectomy. Further studies with larger number of animals are needed to understand the clinical relevance of IC and SC in feline mammary tumours prognosis as complementary techniques to histopathology.

Keywords: feline mammary tumors, imprint cytology, scrape cytology, histopathology, sensitivity, specificity, positive predictive value, negative predictive value, accuracy.

Abbreviations

AFAST - abdominal focused assessment with sonography for trauma, triage, and monitoring

BCS - breast-conserving surgery

FMT – feline mammary tumour

FS – frozen section analysis

IC – imprint cytology

ICU – intensive care unit

IDSM - intraoperative digital specimen mammography

IOUSG - intraoperative ultrasonography

JC – José Catarino

JF – Joana Fonseca

JM – João Martins

LM – Liege Martins

Micro-CT - microcomputed tomography

MRI – magnetic resonance imaging

NIR - near-infrared

NPV – negative predictive value

OCT - optical coherence tomography

PC – Patrícia Cabral

PF – Pedro Faísca

PPV – positive predictive value

SC – scrape cytology

SR – specimen radiography

TECAs – total ear canal ablations

TFAST – thoracic focused assessment with sonography for trauma, triage, and monitoring

ULHT – Universidade Lusófona de Humanidades e Tecnologias

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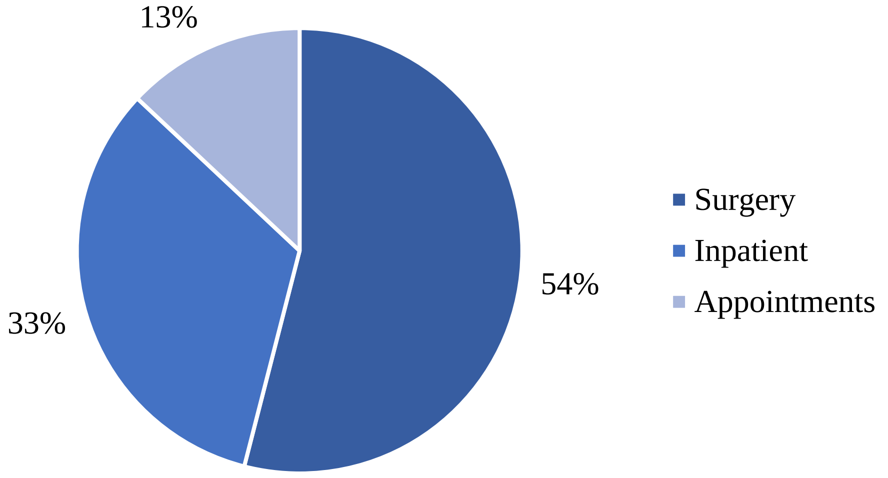
1. Description of the internship caseload

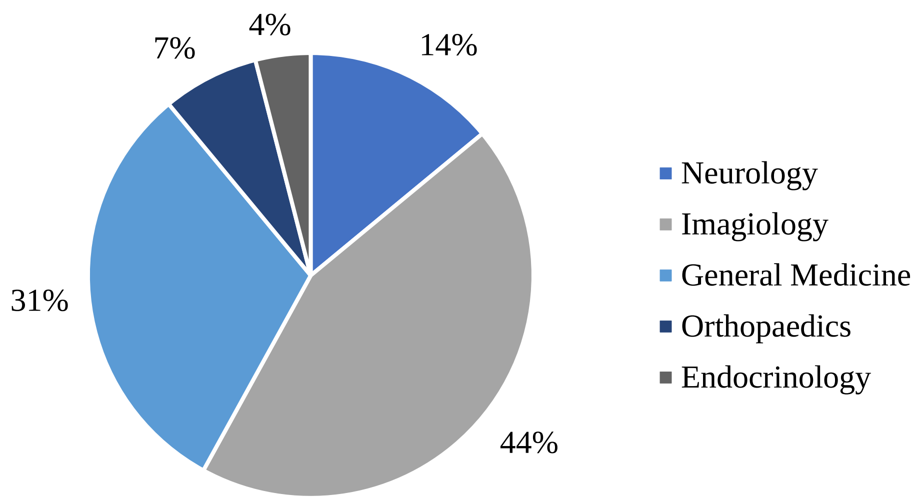
This report is an objective description of the curricular internship carried out by the author at the Veterinary Hospital of Universidade Lusófona (ULHT) from November 1, 2022 to April 30, 2023, with the main focus on pet medicine and surgery. It was a rotational internship between the different hospital areas that included night and weekend shifts. During the internship period, the intensive care unit and the surgery section were the main departments in which the author acquired knowledge, and specialty appointments were also attended, particularly imaging and neurology.

The ULHT Veterinary Hospital has an abundant and varied caseload, with cases being monitored from the moment of admission until discharge, which was a very enriching factor for the author's internship. In addition, as this is a university hospital, the author was constantly accompanied by teachers who gave the opportunity for perfecting skills.

Prior to the start of the internship, the timetable was discussed with the clinical directors and it was agreed that the internship would be rotational, with a greater focus on the surgical area, in accordance with the author's interests. As such, graph 1 shows the distribution among the different hospital areas during the 24 weeks of the internship, and graph 2 shows the different specialties that the author witnessed when present at appointments. It can be observed that surgery was the predominant area, taking up more than half of the internship, corresponding to 13 weeks.



Graph 2. Division between the different hospital areas during the internship.



Graph 2. Division of specialty Appointments.

The ULHT Hospital has two intensive care units that divide the different species, two inpatient units for non-serious cases, also for the two species, and an infectious diseases unit for both species subdivided within it. In graph 1, the "Inpatient" slice corresponds to the intensive care units and the inpatient units. The author rarely worked in the infectious diseases' unit.

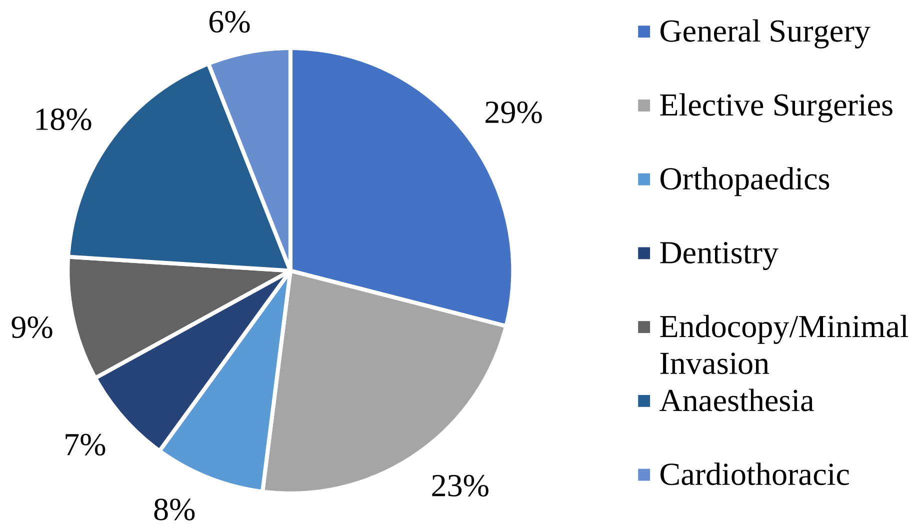
In the intensive care unit, the author rotated between the dog intensive care unit (ICU) and the cat ICU. Here different cases were monitored, physical examinations as well as complementary tests such as radiographs, abdominal focused assessment with sonography for trauma, triage, and monitoring (AFAST) and thoracic focused assessment with sonography for trauma, triage, and monitoring (TFAST), and cystocentesis were performed. Normal ICU duties were also among the author's tasks, such as preparing and administering drugs, changing dressings, drawing blood, placing catheters, placing oesophageal tubes, calculating fluid therapy with or without supplements, and euthanasias. The author occasionally took part in emergency consultations, particularly cases of polytraumatized animals with or without head trauma, dyspnoeic animals, drug ingestions and burns.

Inpatient units for non-serious cases were also areas where the author worked, although less frequently, as these animals require less care and many of them are in "hotel" conditions. Here, the normal caseload is just to carry out less thorough physical examinations and administer medication.

While scheduled to work at the ICUs, the author was able to witness the clinical rounds, where there were discussions about the inpatient cases. In addition, the author was frequently questioned about the clinical cases present. These two factors allowed the author to refine clinical thinking.

During the weeks of specialty appointments, the most frequent were the imaging appointments. These included attending and participating in abdominal ultrasounds, echocardiograms and CT scans, with a frequent task in the latter being the anaesthetic monitoring of sedated animals. General medicine appointments consisted mainly of annual follow-ups of healthy animals, with the administration of vaccines and dewormers, and occasionally the need to prescribe drugs. These also included animals coming in for dermatological complaints. Orthopaedic appointments consisted exclusively of post-surgical fractures, where the author was accompanied by the surgeon and was able to observe the orthopaedic examination and post-surgical progress through X-rays. The neurological appointments were particularly astonishing, as the initial approach was carried out exclusively by the author under the guidance of the neurologist. In these consultations, the author learned how to carry out a correct neurological examination and discussed the possible causes and treatments of the problem in question with the neurologist.

As already mentioned, surgery is the author's main area of interest and took up the largest part of the internship. Graph 3 shows the different sub-areas in which the author participated while in surgery. A distinction was made between general surgery and elective surgery, the latter consisting exclusively of ovariohysterectomies and orchiectomies in small animals, to explain the importance of elective surgeries during the internship. These were carried out by the author, initially as the second surgeon and in the end only under the supervision of the surgeon, which was another strong point of the internship at this institution. It is important to mention that, due to the *trap, neuter, release* protocols that the hospital has with some local municipal councils, the author was able to learn and master how to perform orchiectomies on cats.



Graph 3. Task Division while in surgery.

In general surgery, the author was either the second surgeon or only assisted in the procedure while performing the anaesthetic monitoring. Within this modality, mastectomies in cats stand out, in which the author had a special interest given the dissertation's topic. In this surgical area, the author highlights the participation in cutaneous nodulectomies, enterotomies and enterectomies, gastrotomies to remove foreign bodies, cystotomies, salivary gland excisions, urethrostomies, exploratory laparotomies with biopsy collection, splenectomies, total ear canal ablations (TECAs) and resolution of otohematomas.

Dental, orthopaedic, and cardiothoracic surgeries occupy the smallest shares of the caseload. Orthopaedic surgeries were mainly amputations, with the remaining procedures being osteosynthesis and removal of femoral heads. Dental surgeries consisted of dental scaling and the removal of dental pieces. The hospital has few cardiothoracic cases, but it is an area of interest to the author. Particularly striking procedures seen by the author were the correction of diaphragmatic hernias, both in polytraumatized animals and congenital cases, the correction of persistent ductus arteriosus, and septal defects.

The hospital has a varied caseload in the area of endoscopy/minimal invasion. Of note are ovariohysterectomies in large dogs, bronchoscopies, gastroscopies for foreign body removal and sample collection, vaginoscopies, and thoracoscopies with thoracic duct ligation to resolve chylothorax.

In the area of anaesthesia, the author carried out anaesthetic protocols and monitoring for various procedures, from sedation for ultrasounds on fractious animals to exploratory laparotomies, always under the supervision of an anaesthesiologist. Post-anaesthetic monitoring and the establishment of appropriate post-surgical pain management plans were tasks assigned to the author by the anaesthesiologists and surgeons and, being accompanied at all times, the author was able to make correct choices that provided greater post-surgical comfort to the patient.

The internship, especially in surgery, provided the author with an excellent opportunity to perfect clinical reasoning in the area of greatest interest. The author is better able to correctly identify surgical referral for different conditions, as well as having a more in-depth knowledge of how to perform different surgical procedures in the areas of soft tissue and orthopaedics. In addition, the author was able to deepen the knowledge of endoscopy and minimal invasion surgery, and multimodal anaesthesia with a greater emphasis on loco-regional anaesthesia.

Exotic animal medicine was an area that the author did not delve much in, however, due to the hospital's caseload of these species, the author was able to develop this area, having had the opportunity to learn more about the handling of these species, their particular care, how to perform ovariohysterectomies and anaesthetic plans on rabbits, and to develop basic notions of rabbit dentistry.

The general aim of this internship was to develop the skills acquired during the teaching component of the course, as well as knowing how to apply them in a hospital setting.

It was the author's intention to describe it in a simple, objective and concise way, gathering only the most useful information in order to characterize it effectively. The tasks carried out during the internship were central to the author's professional training, as they are the cornerstones of any veterinary hospital. The author is also aware that the opportunities available in this institution are not common in private hospitals, which is why ULHT Veterinary Hospital was chosen. The author hopes that the expectations of the ULHT Hospital during the 6-month internship were met and concludes that the objectives have been achieved, although with a lot of room for improvement.

2. Introduction

The complete removal of all cancerous tissue is a crucial step to prevent recurrences in oncological surgery (Bray *et al.*, 2023). Over the years, and especially in human medicine, several real time intraoperative margin assessment techniques have emerged (Koopmansch *et al.*, 2021). Frozen section analysis (FS) tended to be the most used method of intraoperative margin assessment, however, due to its many disadvantages, it triggered the search for other simpler methods (D'Halluin *et al.*, 2009; Pradipta *et al.*, 2020). Imprint Cytology (IC) and Scrape Cytology (SC) stand out amongst these, with comparable performances to FS (Pradipta *et al.*, 2020).

Histopathology of a paraffin section is the current gold standard to evaluate surgical margins, however, there are several downsides to this technique arising from only allowing evaluation of a small fraction of the surgical margin (Milovancev *et al.*, 2017). A 2 cm length specimen requires 4000 tissue sections to properly examine all its surface, rendering it impossible for the pathologist, who instead chooses only regions of interest to analyse, resulting in sampling errors (D'Halluin *et al.*, 2009). This further sparked interest in the search for other margin assessment techniques. In veterinary medicine, however, research over this topic remains scarce, and their development could improve patient care (Milovancev *et al.*, 2017).

Feline mammary tumours (FMTs) are characterized by a high frequency of metastatic spread (Chocteau *et al.*, 2019) and are the third most common neoplasms in the intact queen (Cassali *et al.*, 2020). The best approach to cats with FMTs is bilateral mastectomy with lymph node excision, with the possibility of being performed in two different surgical moments (Cassali *et al.*, 2020). Incomplete surgical resection might occur in 45.6% of feline mastectomy patients (Chocteau *et al.*, 2019) and, despite proper surgical planning, intraoperative margin assessment techniques may positively influence patient's life span.

2.1 Margin Assessment Techniques

The main goal of cancer surgery is to achieve a microscopic resection free of cancer cells, in other words, to completely remove the tumour from the remaining wound bed (Bray *et al.*, 2023). A margin is defined as the distance between the excised tissue edge and any tumoral tissue (Bakhshandeh *et al.*, 2006; Bray *et al.*, 2023), and positive margins are responsible for a local recurrence rate of up to 25% 3 to 5 years after human breast-conserving surgery (BCS) as opposed to 3.7% when the margins are negative (Valdes *et al.*, 2007).

In human medicine, the employment of intraoperative pathological diagnostic methods are crucial foundations to obtain clean surgical margins in BCS, culminating in reductions of recurrence rates (Pradipta *et al.*, 2020). FS is the most used method for intraoperative evaluation of surgical margins in human medicine, however, it presents several disadvantages, it is time consuming, costly, and does not perform well on adipose tissue, rendering it especially ineffective in BCS (D'Halluin *et al.*, 2009; Pradipta *et al.*, 2020).

As such, there has been a gradual shift in the search for alternative intraoperative margin assessment techniques, with two of the most promising being IC and SC (D'Halluin *et al.*, 2009; Kolte & Satarkar, 2010; Pradipta *et al.*, 2020).

2.1.1. Frozen Section Analysis

FS of intraoperative surgical margins is uncommon in veterinary medicine. Although accurate, there has been only one published study about this technique in veterinary medicine (Whitehair *et al.*, 1993), and in human medicine itself it is falling into disuse due to its many disadvantages (D'Halluin *et al.*, 2009). There is evidence that the employment of any cytological method in conjunction with FS yields superior results than those obtained by either method alone (Scucchi *et al.*, 1997), however, others concluded that there was a statistically insignificant increase in diagnostic accuracy when cytology and FS were used together (Shidham *et al.*, 2000). Additionally, the quality of cytological preparations can be superior to those of FSs, as the latter frequently suffers from freezing artifacts (Mair *et al.*, 1991; Shidham *et al.*, 2000).

IC and SC seem to be comparable techniques to FS, with both frozen section and cytological examination alone having an acceptable rate of diagnosis, ranging from 93% to 97% (Khalid & Haque, 2004; Kolte & Satarkar, 2010; Esbona *et al.*, 2012).

2.1.2. Imprint Cytology

Cox *et al.*, 1991 were the first to implement imprint (Figure 1.) in the intraoperative evaluation of surgical margins of breast lumpectomy specimens in human patients, with encouraging results, as there was an overall diagnostic accuracy of 97.3%. D'Halluin *et al.*, 2009 conducted a comparative study between intra-surgical imprint cytology and post-surgical histological analysis of 400 BCS procedures in women. They determined that between the two methods there was a correlation of 91.5%, with a sensitivity of 88.6% and specificity of 92.2%, the positive predictive value (PPV) and negative predicative value (NPV) being 73,6% and 97,0%, respectively. There seems to be a tendency for higher sensitivities in invasive lesions (D'Halluin *et al.*, 2009). This could be explained as surgeons perform wider resections on palpable lesions rather than nonpalpable lesions, the latter being frequently associated with in situ carcinomas (D'Halluin *et al.*, 2009). Furthermore, this new method only took, on average, 10 minutes to perform. This technique avoided a second surgical procedure to correct narrow margins in 11.75% of cases. Other studies performed in human patients obtained results that do not veer from these, with sensitivities and specificities up to 97% and 100% respectively, and PPV and NPV up to 100% and 99% respectively (Klimberg *et al.*, 1998; Bakhshandeh *et al.*, 2006).

Figure 2. IC is performed by pressing the glass slide on the fresh cut surface of the specimen (Milovancev *et al.*, 2017).

Table 1 aggregates the results of five of the most promising studies regarding IC in BCS. A pattern of lower PPVs can be observed, explained by slide interpretation errors leading to an elevated number of false positives, particularly in the presence of fibrocystic mastopathy lesions (D'Halluin *et al.*, 2009). Higher false positive rates are a prevalent problematic in human medicine. Recurrence rates diminish with larger volume excisions, while negatively affecting cosmetic results. BCS's objectives are, therefore, to excise the least amount of cancerous tissue that will lead to negative margins, maximize the cosmetic result, and minimize psychological issues that arise from large volume excisions (Valdes *et al.*, 2007). Cosmetic result, however, does not tend to be a concern of pet owners.

Table 2. Comparison of the accuracy of imprint cytology in the intraoperative assessment of margin status.

			Imprint Cytology			
	No. of specimens	**N. of slides**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
Bakhshandeh *et al.*, 2006	100	510	97	99	84	99
Cox *et al.*, 1999	114	----	100	96.6	88	100
Creager *et al.*, 2002	141	758	80	85	40	97
D'Halluin *et al.*, 2009	400	1665	88.6	92.2	73.6	97.0
Klimberg *et al.*, 1998	428	----	93.39	100	100	99.3

"PPV" – Positive Predictive Value; "NPV" – Negative Predictive Value.

The guiding principle of this technique is that, in the presence of malignant cells, these tend to adhere to the glass slide, while adipose cells do not (Valdes *et al.*, 2007). False negatives could, therefore, be explained by failure of these cells to adhere to the glass slides, or the presence of tissue folds that conceal tumoral lesions (Creager *et al.*, 2002; D'Halluin *et al.*, 2009). Nevertheless, false negatives are rare (D'Halluin *et al.*, 2009), with NPVs over 97% (Cox *et al.*, 1991; Klimberg *et al.*, 1998; Creager *et al.*, 2002; Bakhshandeh *et al.*, 2006; D'Halluin *et al.*, 2009).

The main criticism of IC is its inability to assess the safety limits of margins (D'Halluin *et al.*, 2009). D'Halluin *et al.*, 2009 explains that this is circumvented, since pressing the glass slide against the specimen causes malignant cells to seep out of the infiltrated ducts or vessels, who subsequently adhere easily to the slide. They also present their 1.25% re-excision rate as proof that, while IC cannot give a quantitative value of the safety margin limit, it can, through the above-mentioned phenomena, indirectly provide information regarding it. The accuracy of IC varies between tumour types, round cell tumours may exfoliate easily, while sarcomas do not (Milovancev *et al.*, 2017).

IC has several advantages: it allows complete microscopic analysis of the entire specimen (D'Halluin *et al.*, 2009), since most non-cytological methods only allow 10 to 15% of the specimen surface (Cox *et al.*, 1991; Klimberg *et al.*, 1998, 1999), it is rapid, inexpensive, easy to perform, and does not affect the final histological examination (Bakhshandeh *et al.*, 2006; D'Halluin *et al.*, 2009). Additionally, it can overcome the shortcomings of frozen section analysis, since in this latter technique it is impractical to evaluate the whole surface margin (Creager *et al.*, 2002) and can potentially compromise the final diagnosis by loss of small lesions (Klimberg *et al.*, 1998). Weinberg *et al.*, 2004 also concluded that IC diminished the incidence of 5-year local recurrence from 8.8% to 2.8% when compared to frozen section. Nevertheless, Tamanuki *et al.*, 2021 concluded that IC followed by frozen section is the best method to determine intraoperative margins, since it markedly decreases positive margin rates.

2.1.3. Scrape Cytology

SC is a modification of IC (Sharma *et al.*, 2021). Whereas in IC a slide is gently pressed along the surface of the excised specimen, in SC the wound bed left after excision is lightly scraped, with the collected material smeared on a slide afterwards (Milovancev *et al.*, 2017; Sharma *et al.*, 2021). Several studies have reported high concordance between this technique and histopathology, ranging from 93% to 97% depending on the study (Kolte & Satarkar, 2010; Mahore *et al.*, 2015; Sharma *et al.*, 2021).

Kolte & Satarkar, 2010 assessed the diagnostic capabilities of SC in a myriad of tumour types, having obtained a 97.3% accuracy rate when compared to histopathology. Similarly, both Mahore *et al.*, 2015 and Sharma *et al.*, 2021 tested SC in the diagnosis of tumours and obtained accuracies of 93.49% and 96%, respectively.

Of the 169 specimens analysed in Mahore *et al.*, 2015, 18 originated from breast tissue and there was 100% concordance with histopathology. Similarly, in Kolte & Satarkar, 2010, of the 75 specimens, 40 came from breast tissue and 39 were correctly diagnosed, making the concordance rate between SC and histopathology 97.5%.

Low-grade or well-differentiated lesions contribute to lower diagnostic accuracies, and accuracies for this technique can vary considerably given the types of tumours encountered (Kolte & Satarkar, 2010). Nevertheless, accuracies between the published literature correlate with each other. As can be observed in table 2, that aggregates different studies, accuracies vary between 93.49% to 97.8%.

Table 2. Diagnostic utility and accuracy of scrape cytology in different types of tumours.

			Scrape cytology				
	No. of specimens	**No. of slides**	**Sensitivity (%)**	**Specificity (%)**	**Accuracy (%)**	**PPV (%)**	**NPV (%)**
Gautam *et al.*, 2021	50	----	90	100	97.8	100	97.4
Kolte & Satarkar, 2010	75	≈4/specimen	----	----	97.3	----	----
Mahore *et al.*, 2015	169	3-4/specimen	----	----	93.49	----	----
Mair *et al.*, 1991	206	----	----	----	97.6	----	----
Sharma *et al.*, 2021	50	4-5/specimen	90.9	97.4	96	90.91	97.44
Shidham *et al.*, 2000	446	----	91.2	96.9	93.7	----	----

“PPV” – Positive Predictive Value; “NPV” – Negative Predictive Value.

Unlike IC, very few studies have been published that evaluate SC in the assessment of BCS margins. The results of the only two articles regarding this topic are presented in table 3. Both studies obtained similar results, with high sensitivity, specificity, and accuracy in the assessment of margin status in lumpectomy specimens.

Table 3. Comparison of the accuracy of scrape cytology in the intraoperative assessment of margin status.

			Scrape cytology		
	No. of specimens	**No. of slides**	**Sensitivity (%)**	**Specificity (%)**	**Accuracy (%)**
Gal, 1988	30	----	100	96	96.7
Tohnosu *et al.*, 1998	50	200	96.4	90.7	91.5

According to Kolte & Satarkar, 2010, scraping the tumour is a preferable method to IC, as a larger number of cells can be obtained in the former method. In a study that aimed to assess the role of IC and SC in the diagnosis of soft tissue tumours with posterior comparison with histopathology, IC alone revealed to not be very helpful in the intraoperative diagnosis (Gautam *et al.*, 2021). SC had an accuracy of 97.8% and a diagnostic yield of 100%, while IC only had an accuracy of 22% with a diagnostic yield of 24%.

The drawbacks of SC are similar to those of IC, as it is unable to provide information regarding the depth of invasion, margin safety and the presence of residual disease beneath the surface (Kolte & Satarkar, 2010; Butler-Henderson *et al.*, 2014; Sharma *et al.*, 2021). Butler-Henderson *et al.*, 2014 considered the time required for cytological preparations too long, with the possibility of leading to additional time under anaesthesia.

SC possesses high accuracy rates, with preservation of cellular details while not sacrificing tissue needed for the histology diagnosis, it is a simple, inexpensive and quick method (Shidham *et al.*, 2000; Kolte & Satarkar, 2010; Sharma *et al.*, 2021).

2.1.4. Emerging Techniques

Other methods of intraoperative margin assessment have recently surfaced, predominantly in human medicine (Pradipta *et al.*, 2020). Grouped within these there are conventional imaging methods and newly emerging diagnosis methods (Pradipta *et al.*, 2020).

Specimen Radiography (SR) is used for the immediate assessment of excised tissue samples (Pradipta *et al.*, 2020). In conventional SR, radiographies of a specimen without palpable lesions are taken in order to exploit the intrinsic characteristics of this method, namely the different contrasts obtained after beam attenuation through different tissues (Pradipta *et al.*, 2020). However simple this method may be, to the authors knowledge, no studies have been conducted in veterinary medicine. Similar to this technique, intraoperative digital specimen mammography (IDSM) serves comparable purposes, providing the added advantage of immediate specimen analysis in the operating room rather than requiring transportation to the radiology room, as occurs in conventional SR (Pradipta *et al.*, 2020).

Intraoperative Ultrasonography (IOUSG) allows visualization of the to be excised tissue preoperatively, with posterior ex vivo ultrasonography to verify the correct removal of all targeted areas (Pradipta *et al.*, 2020). Shores *et al.*, 2021 studied the applications of IOUSG in the operating room to identify, localize, and monitor the removal of intracranial lesions, including tumours, in small animals, with favourable results (Figure 2).

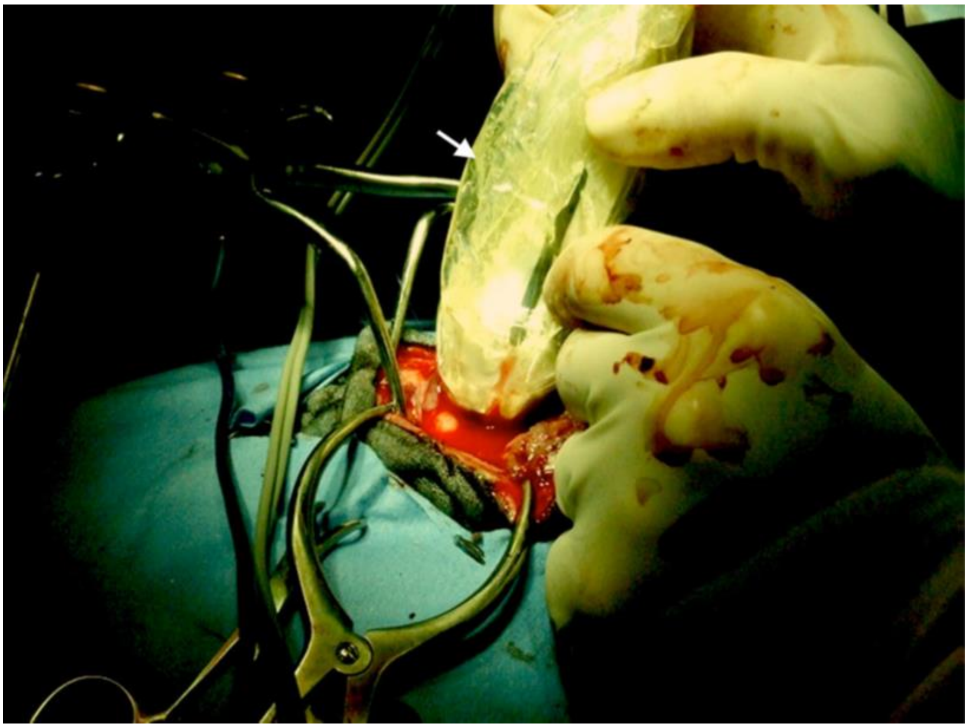


Figure 2. Intraoperative photo showing ultrasound transducer covered with a sterile sleeve (white arrow) and applied directly to the cerebral cortex during a rostrotentorial craniectomy. (Shores *et al.*, 2021).

Newly emerging methods based on computed tomography, magnetic resonance imaging (MRI), spectroscopy, and chemical approaches, are being developed in order to overcome the shortcomings of pathological analysis (Pradipta *et al.*, 2020).Optical coherence tomography (OCT) functions similarly to ultrasound imaging, in this case using light waves instead sound waves. Through the application of near-infrared light, imaging of up to 2 mm beneath the tissue surface can be obtained and allows differentiation of normal mammary gland tissue from tumoral tissue (Pradipta *et al.*, 2020). OCT is not routinely used in veterinary medicine, however, its applications have been studied in eighty dogs undergoing excision for cutaneous and subcutaneous tumours (Figure 3), with overall median sensitivity and specificity for detection of incomplete margins of 86.7% and 84.6%, respectively (Cheng *et al.*, 2022).

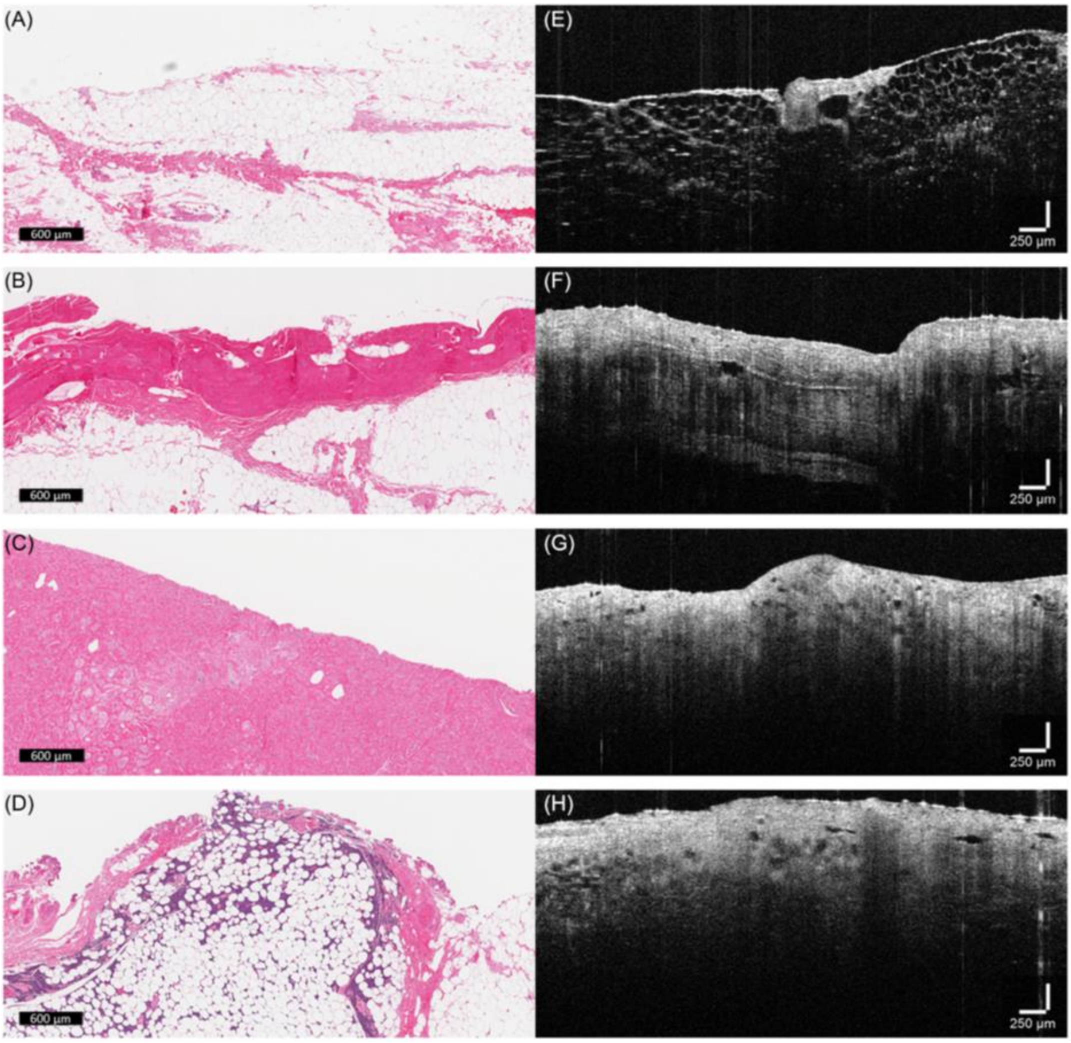


Figure 3. OCT allows differentiation between normal tissue from tumoral tissue. These images showcase the correlations between different tissues on haematoxylin and eosin stained histology slides and their corresponding OCT images. A and E correspond to adipose tissue, B and F to skeletal muscle, C and G to fibroma, and D and H to subcutaneous mast cell tumour (Cheng *et al.*, 2022).

Microcomputed Tomography (Micro-CT) allows measuring tumours in three dimensions in entire live BCS specimens, with higher accuracy when compared to preoperative mammography, ultrasound, MRI, and histopathology (Pradipta *et al.*, 2020). Micro-CT has been researched in veterinary medicine, especially for the diagnosis of oral disease in rabbits (de Rycke *et al.*, 2012; Sasai *et al.*, 2014). Eberspächer-Schweda *et al.*, 2020 compared the diagnostic yield of micro-CT to histopathology in canine oral fibrosarcoma, concluding that this novel technique has the potential to support histopathology (Figure 4).

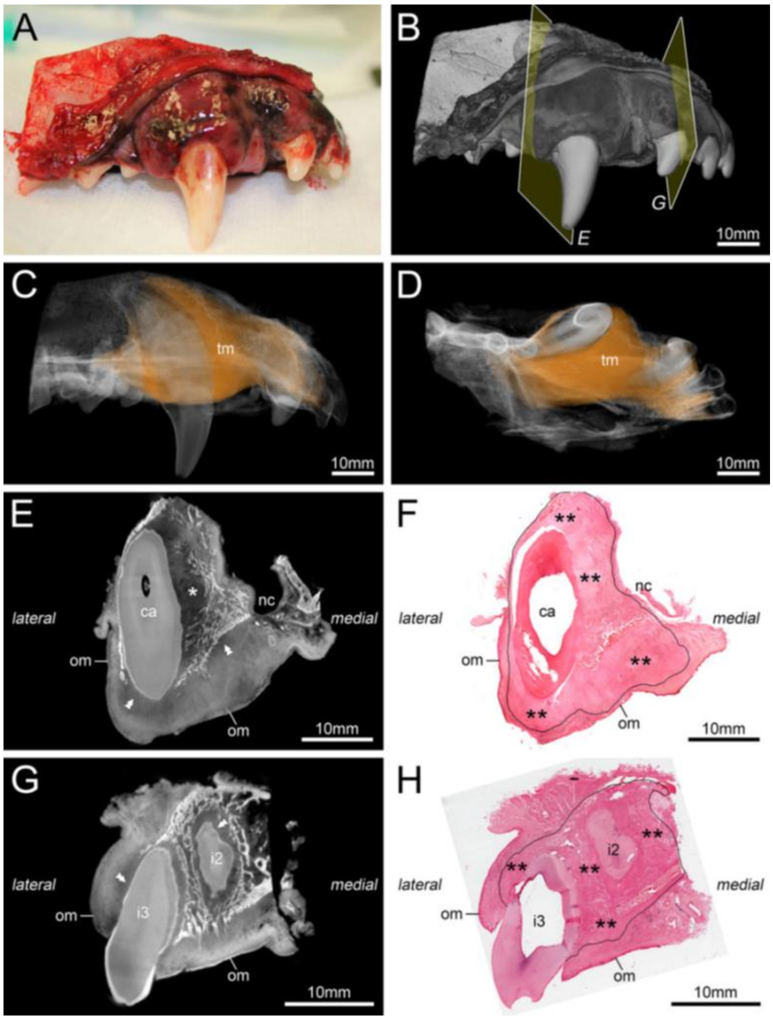


Figure 4. Comparison of micro-CT slices with haematoxylin and eosin stained histology slides in a maxillectomy specimen. (A) Lateral view toward the maxillectomy specimen. (B) Volume rendering of the maxillectomy specimen based on micro-CT image volume. (C and D) Transparent volume renderings depicting the tumour mass (orange volume) based on image segmentation. (E and F) Corresponding micro-CT and histopathological section. The micro-CT image shows severe loss of bone (asterisk) and lower staining intensities (double arrowheads) close to the canine tooth root. Histopathological evaluation allowed unambiguous identification of the tumour margin (black contour) and the tumour mass (double asterisk). (G and H) Corresponding micro-CT and histological section (Eberspächer-Schweda *et al.*, 2020). “ca” – canine; “i2” – second incisor; “i3” - third incisor; “nc” – nasal cavity; “om” – oral mucosa; “tm” – tumour mass.

There has been development of two portable devices capable of margin detection based on spectroscopy (Pradipta *et al.*, 2020). Radiofrequency Spectroscopy measures the local electrical properties of lumpectomy margins and, since normal and tumoral cells have different electrical properties, these can be differentiated (Pradipta *et al.*, 2020). Bioimpedance Spectroscopy functions similarly, in this case detecting differences in tissue dielectric properties (Pradipta *et al.*, 2020). The applications of these technologies have yet to be researched in veterinary medicine.

Raman spectroscopy measures the vibrational frequencies of molecules in tissues that can be excited by laser (Pradipta *et al.*, 2020).It was first developed has a means to diagnose malignant and benign breast lesions, with the possibility of being used as a margin detection method afterwards (Birtoiu *et al.*, 2016). By itself, it cannot image small areas of residual cancer with sufficient accuracy within the limited timeframe possible for intraoperative diagnosis (Pradipta *et al.*, 2020). However, it can be combined with autofluorescence microscopy to overcome this downside, this combination being called Multimodal Spectral Histopathology (Figure 5) (Pradipta *et al.*, 2020). It has recently been applied for the intraoperative differentiation between normal brain tissue from neoplastic brain tissue in dogs (Figure 6), with encouraging results, more studies needing to be carried out in order to properly assess this technology for the intraoperative guidance of tumour resection (Doran *et al.*, 2022).

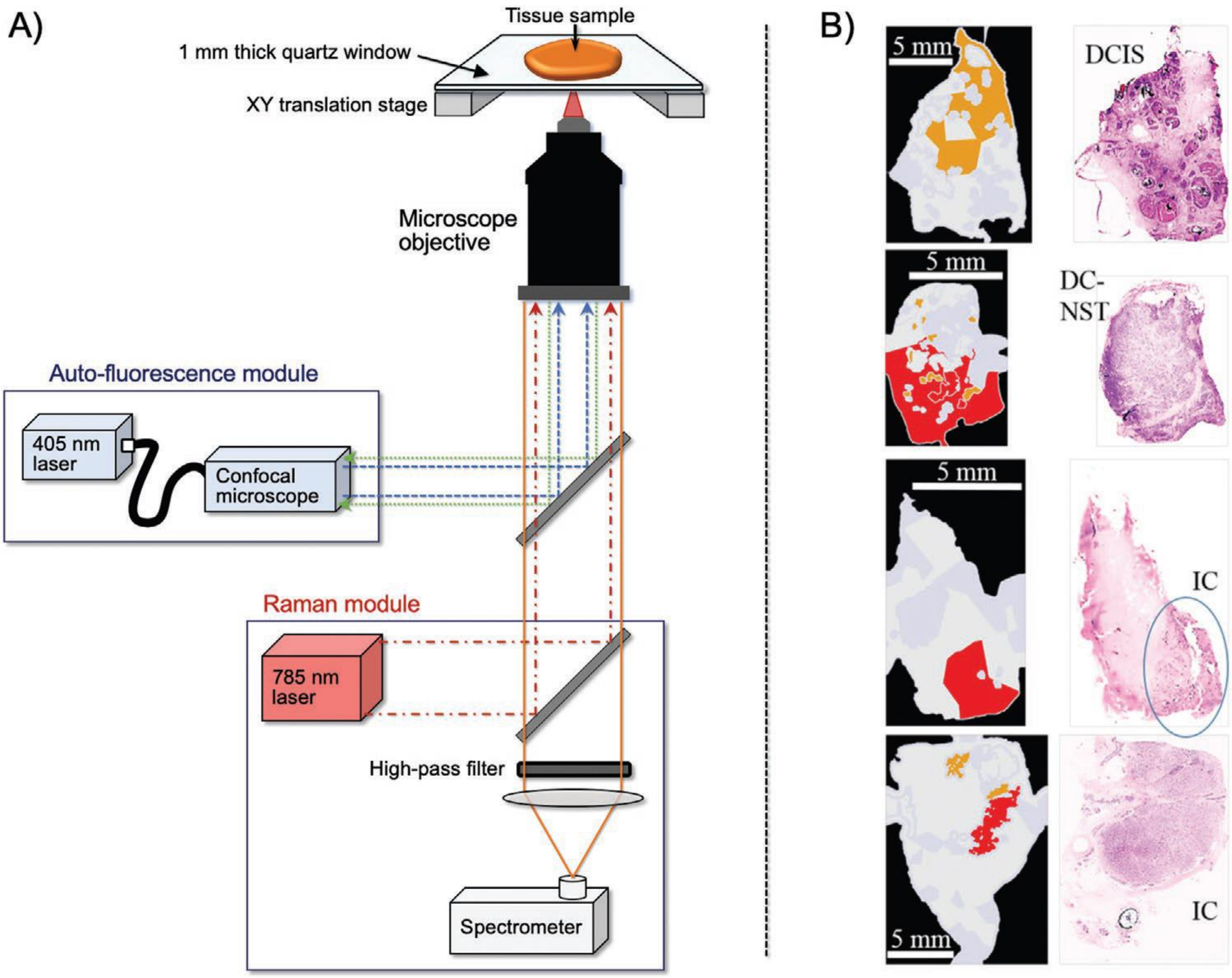


Figure 5. (A) The multimodal spectral histopathology instrument consists of an inverted optical microscope with an integrated Raman spectrometer and a confocal autofluorescence module. (B) Images of breast cancer tissue detected with multimodal spectral histopathology. The corresponding histologic images with hematoxylin and eosin staining confirmed the presence of positive margins in the human patient (Pradipta *et al.*, 2020). "IC" – invasive carcinoma; "DCIS" - ductal carcinoma in situ; "DC-NST" - ductal carcinoma of no special type.

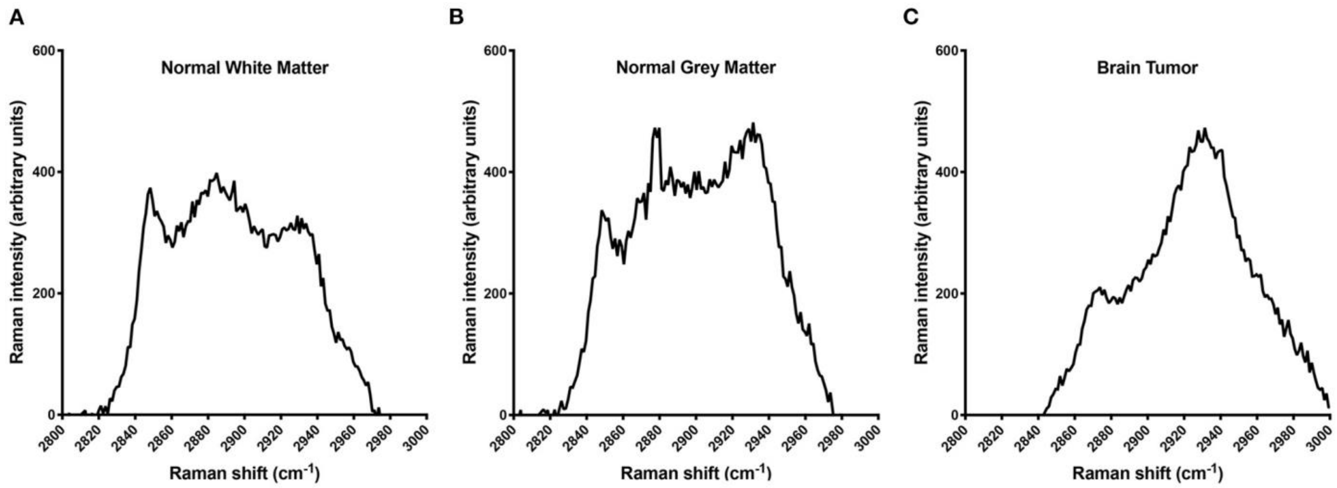


Figure 6. Raman spectra illustrating the differences in the protein: lipid ratio among normal white matter (A), normal gray matter (B), and brain tumour (C) in dogs (Doran *et al.*, 2022).

Chemical probes used to diagnose cancer based in fluorescence properties are under development (Pradipta *et al.*, 2020). Some of these probes “switch-on” either through the detection of cancerous enzymatic activity or upon cancerous protein binding (Figure 7) (Pradipta *et al.*, 2020). Near-infrared (NIR) fluorescence imaging allows, after the administration of a chemical probe, real-time visualization of the excited fluorescent agents present in tumoral tissue (Favril *et al.*, 2018). Research over this topic in veterinary medicine is minimal, however, with encouraging results, being an important candidate technique for sentinel lymph node mapping and tumour margin detection (Favril *et al.*, 2018).

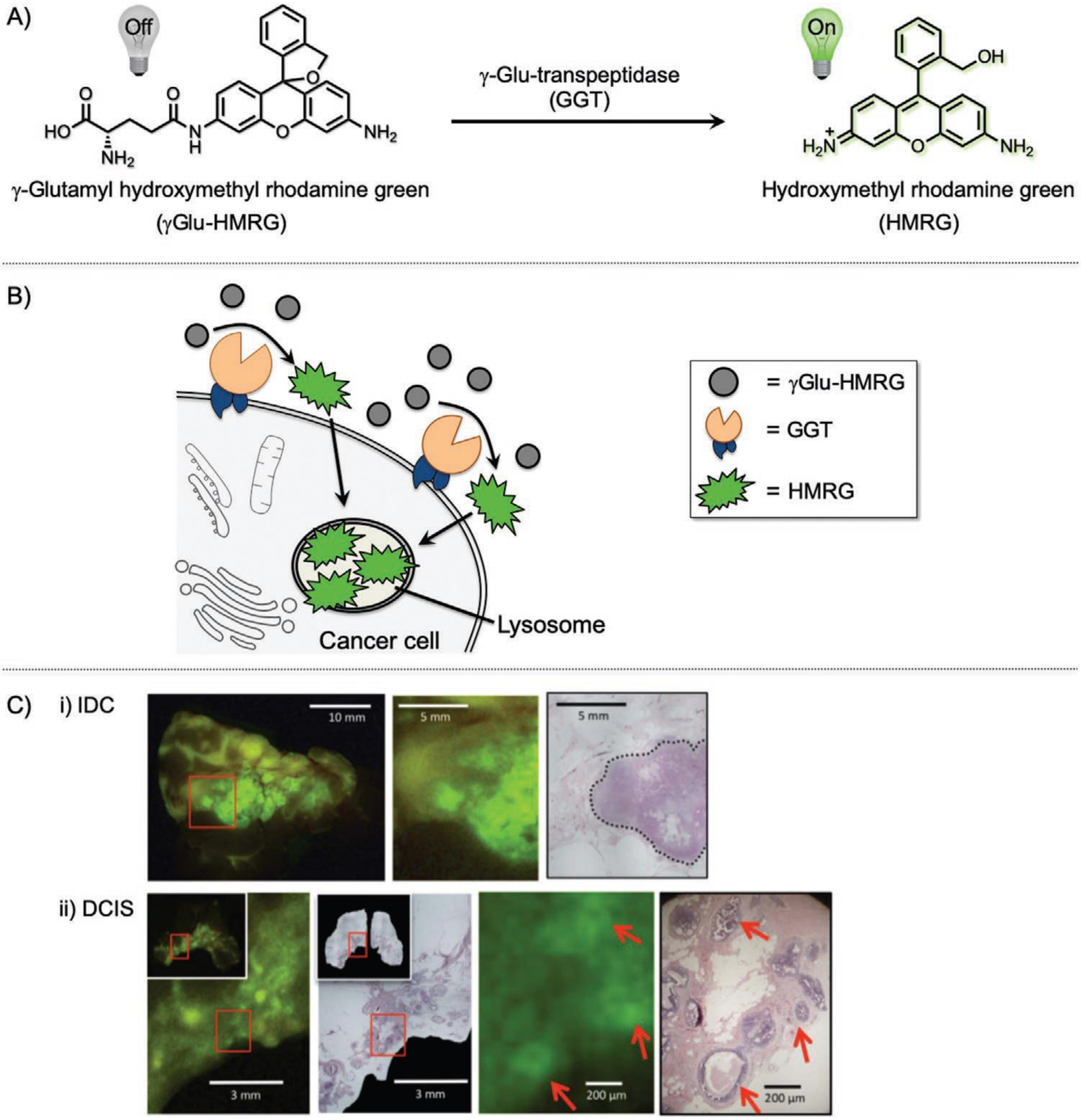


Figure 7. (A) Explicit activation of the γ-glutamyl hydroxymethyl rhodamine green (γGlu-HMRG) probe by γ-glutamyltranspeptidase (GGT). (B) Mechanism of γGlu-HMRG activation - γGlu-HMRG reacts with upregulated GGT anchored on the plasma membrane of cancer cells. This interaction enzymatically cleaves the probe to yield a highly fluorescent and membrane-permeable product, hydroxymethyl rhodamine green (HRMG), which accumulates in the lysosomes of cancer cells. HMRG does not covalently attach to the inside of the lysosome; thus, it can be excreted out of the cell again and affect the surrounding cells. (C) Comparison of fluorescence localization with pathologic hematoxylin and eosin staining. (C-i) The cancer region is enclosed by a dotted line in the image with hematoxylin and eosin staining. (C-ii) Red arrows show fluorescence-positive areas and malignant lesions (Pradipta *et al.*, 2020).

These methods are, even in human medicine, relatively under investigated. They show great promise, however, with superior results than those obtained by frozen section and cytological analysis in some cases. Further research could prove them to be the best choice to reduce margin positivity and, subsequently, diminishing the risk of recurrence.

2.1.5 Histopathology

The theory behind the employment of intraoperative margin assessment techniques is that they provide an indication for the likelihood of tumour recurrence (Pradipta *et al.*, 2020), and, a histological tumour free margin is considered the best predictor of local tumour control (Bray *et al.*, 2023). According to Bray *et al.*, 2023, however, the presence or absence of tumoral cells is not as good an indicator for tumour recurrence as previously thought, as even if histopathology detects tumour cells at the resection margin, tumour recurrence might not occur. The justification for this apparent contradiction lies behind the inherent limitations of histology, which is influenced by variations in tissue elasticity, tissue fixation, histological processing, and the interpretative process itself (Bray *et al.*, 2023).

Histological processing significantly distorts tissue dimensions, with not only the specimen's margins suffering wild variation of their original measures, but also this distortion may misplace the tumour and deep fascial plane (Bray *et al.*, 2023).This disruption can result in both false negatives and false positives, and inking the tissue barriers is advised to overcome these problems (Bray *et al.*, 2023).

Estimates state that only 0.01% to 0.1% of the total circumferential surgical margin can be evaluated through histology (Bray *et al.*, 2023). This proportion can be increased by examination of more sections or employing a different method of margin analysis. Complete bread loafing histopathology increases the percentage of marginal tissue examined when compared to other techniques and provides better detail over the numeric margin distance of the tumour (Kamstock *et al.*, 2011; Cassali *et al.*, 2020).

2.2 Feline Mammary Tumours

Feline mammary tumours (FMTs) are characterized by a high frequency of metastatic spread (Chocteau *et al.*, 2019) and are the third most common neoplasms in the intact queen (Cassali *et al.*, 2020). They constitute approximately 17% of all feline neoplasms and 80-90% are hormone-independent carcinomas of the simple type that have an aggressive biological behaviour (Zappulli *et al.*, 2015). At least 80% of FMTs are malignant, with lymph node and lymphatic vessel invasion being frequent, culminating in more than 80% of cases presenting regional and/or distant metastasis (Cassali *et al.*, 2020). The most common sites of metastasis are regional lymph nodes (83%), lungs (83%), liver (25%), and pleura (22%) (Zappulli *et al.*, 2015). The mean age for the development of FMTs is between 10 to 12 years of age and there is breed predisposition, with an increased risk for Siamese cats for the development of FMTs (Zappulli *et al.*, 2015). Additionally, Siamese cats possess an increased risk for the development of FMTs at a younger age (Zappulli *et al.*, 2015). The prognosis is unfavourable with the mean survival time being less than 1 year (Cassali *et al.*, 2020). Longhair breeds (Siamese and Persian) have a poorer prognosis when compared to all other breeds (Zappulli *et al.*, 2015). Tumour grading is one of the most reliable prognostic indicators (Zappulli *et al.*, 2015). Smaller tumour sizes correlate with better prognosis than larger tumours, and lymphovascular invasion and lymph node involvement are also important prognostic parameters (Zappulli *et al.*, 2015).

Histological grading is based on tissue architecture (tubular formation), cell pleomorphism and mitosis count, always considering invasive areas for evaluation (Cassali *et al.*, 2020). Clinical staging is based on the TNM classification of malignant tumours system developed by the World Health Organization and modified for FMTs (Table 4) (Zappulli *et al.*, 2015; Cassali *et al.*, 2020). Clinical staging permits establishment of prognosis and treatment plan (Cassali *et al.*, 2020).

Table 4. World Health Organization modified clinical staging for feline mammary tumours, according to the TNM system (Adapted from Cassali *et al.*, 2020).

T – Primary Tumour	
T_1	< 2 cm maximum diameter
T_2	2-3 cm maximum diameter
T_3	>3 cm maximum diameter
N – Regional lymph nodes	
N_0	No metastasis (histology or cytology)
N_1	Metastasis present (histology or cytology)
M – Distant metastasis	
M_0	No distant metastasis detected
M_1	Distant metastasis detected
Stages	
I	$T_1N_0M_0$
II	$T_2N_0M_0$
III	$T_{1\text{-}2}N_1M_0$
	$T_3N_{0\text{-}1}M_0$
IV	Any T $N_{0\text{-}1}M_1$

The main treatment is surgical excision, the preferred method being two-step bilateral mastectomy, since the recurrence rate for this technique is far lower (20%) than when unilateral mastectomy is performed (46.7%), as is observed in the rate of distant and regional metastasis as well (Gemignani *et al.*, 2018; Cassali *et al.*, 2020). In addition, regional, axillary, and inguinal lymph nodes should always be excised, regardless of the presence or absence of any clinical alterations (Cassali *et al.*, 2020). The high frequency metastatic spread with 80% of cases being malignant and unfavourable prognosis pose a concern for veterinary oncologists and surgeons regarding the diagnostic accuracy and therapeutical strategies (Cassali *et al.*, 2020). A clean surgical margin is associated with longer disease free intervals and overall survival (Gemignani *et al.*, 2018; Chocteau *et al.*, 2019). The adaptation of the intraoperative margin assessment methods frequently utilized in human BCS could prove an advantage in the management of this disease.

2.3 Intraoperative Cytology in Veterinary Medicine

There has been only one study conducted to evaluate the applicability of intraoperative cytology in veterinary medicine (Milovancev *et al.*, 2017). Their objective was to determine the agreement between margin status diagnosed on IC, shaved margin histopathology, and radial section histopathology in canine cutaneous and subcutaneous mast cell tumours and soft tissue sarcomas. They concluded that, although feasible, there was poor agreement between methods. They explain that each technique will provide different information depending on the clinical scenario, as was observed in the study when IC yielded higher positive margin rates than the other methods in mast cell tumours.

One of the major pitfalls associated with shaved margin histopathology is that it cannot always be used without increasing the risk of patient morbidity, as it can increase the risk of wound healing complications or critical anatomic structures can me mistakenly incised (Milovancev *et al.*, 2017). In the case of Milovancev *et al.*, 2017, shaved margin samples could not be collected in 5% of cases because of the presence of vital structures at the edges of the wound bed.

The objective of the present study is to determine the applicability of IC and SC techniques during the intraoperative period and their diagnostic accuracy for margin status assessment when compared to histopathology in unilateral radical mastectomy for FMT surgical treatment.

3. Materials and methods

3.1. Study population

Twelve client-owned female cats, requiring mastectomy as surgical treatment, were recruited. The study was carried out over a consecutive 16-month period (February 1, 2022 – May 31, 2023) and took place at the veterinary teaching hospital of the Faculty of Veterinary Medicine, Lusófona University, in Lisbon, Portugal. Ethical approval was obtained from the Comissão de Ética e Bem-Estar Animal (CEBEA) from the Faculty of Veterinary Medicine of the Lusófona University (17-2022). Written informed consent was obtained from all owners. Cats undergoing surgery, were previously evaluated by an oncologist and underwent oncological staging. No breed or weight criteria were used. All surgeries were performed by 3 experienced surgeons (JM, PC or LM). Eleven unilateral radical mastectomies and one partial cranial unilateral mastectomy were included.

Exclusion criteria included animals with detectable tumours other than mammary tumours adjacent to the mammary chain or close or within the delineated margins of the surgical field.

3.2. Sample collection

Since mastectomy specimens and operative field surfaces measure more than 5 cm, in order to standardize and simplify intraoperative sample collection, both surfaces were divided in cranial surface (first and second mammary gland) and caudal surface (third and fourth mammary glands) (Figure 8.).

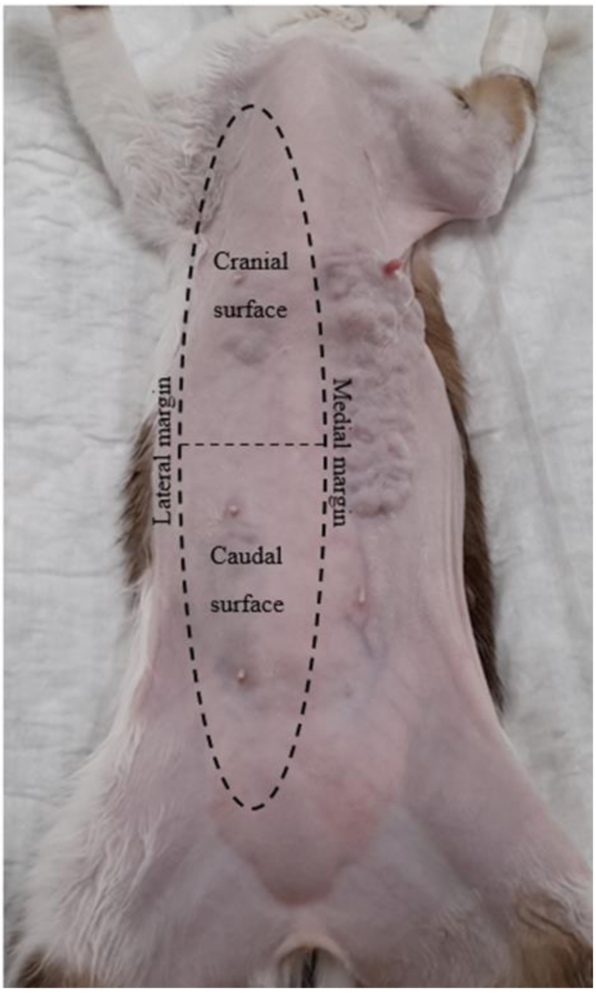


Figure 8. Illustration of mastectomy divisions in cranial and caudal surface and margins in order to perform scrape cytology in the surgical field, and imprint cytology and complete bread loafing section histopathology in the mastectomy specimen (original).

Samples from the cranial and caudal aspects of the mammary gland, were collected from the medial, lateral and deep margin surfaces, resulting in 6 glass slides to cover the entire mammary gland's margins and 6 glass slides to cover the surgical field (Figure 9.).

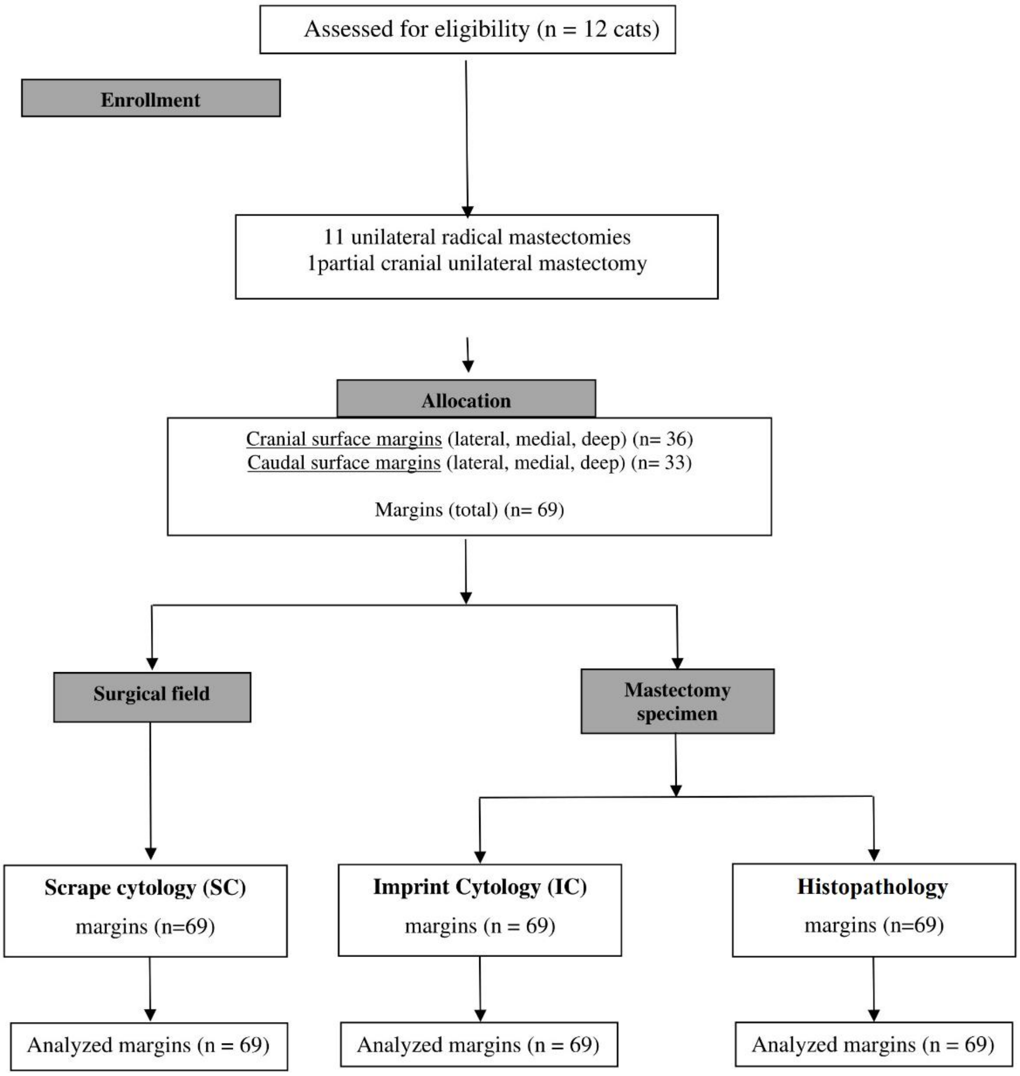


Figure 9. Schematic diagram of sample collection.

3.3. Imprint cytology

All imprints were identified and prepared from each *ex vivo* mastectomy specimen after surgical excision. Each identified glass slide was gently pressed against each freshly cut mastectomy specimen margin of interest (eg. Cranial lateral, medial and deep or caudal lateral, medial and deep).

3.4. Scrape cytology

All scrapes were identified and prepared from the surgical field margins with a gentle # 24 scalpel blade scraping to each surface of interest (eg. Cranial lateral, medial and deep or caudal lateral, medial and deep) and smeared on a glass slide.

All glass slides obtained from IC and SC methods were allowed to air dry and stained manually with a rapid aqueous based Romanowsky stain (Hemacolor®, Merck KgaA, Darmstadt, Germany). Cytology samples were analysed by an experienced pathologist (JF), after the surgical procedure and unaware of the histopathology margin status. Additionally, indication of the precise site of the lesion were described: cranial and caudal aspects of lateral, medial or deep regions.

3.5. Histopathology

The excised mastectomy specimens were identified with sutures (Kamstock *et al.*, 2011) in 4 directions: (cranial- 1 suture, caudal- 2 sutures, lateral- 3 sutures, medial-4 sutures).

After 10-25 minutes of air drying at room temperature and after suturing and collecting samples for IC, mastectomy specimens were immersed in 10% neutral buffered formalin at a ratio of 1:10 tissue/formalin volumetric ratio, and submitted for routine histopathology.

The samples of the study were trimmed and evaluated by two anatomic pathologists (PF and JC). In order to confirm the diagnosis and quantify the histologic tumour-free margin of the areas of interest (cranial lateral, medial and deep or caudal lateral, medial and deep), complete bread loafing sections were trimmed. All trimmed samples were processed for routine histopatology at the Laboratório de Análises Clínicas e Histológicas Veterinárias in Lusófona University. Feline mammary tumours were evaluated and graded based on established prognosis criteria tissue invasion, cellular and nuclear pleomorphism, degree of anisocytosis and anisokaryosis, nucleolar features, mitotic index, necrosis, and lymphovascular invasion (Kamstock *et al.*, 2011).

All histopathology margin status were analysed by the two anatomic pathologists, unaware of the intraoperative cytology method's margin status.

3.6. Evaluation of margin status

For each mastectomy specimen, individual margins were evaluated in the areas of interest by IC, SC, and complete bread loafing section histopathology, and determined as positive or negative for tumour cells. An IC and SC margin was defined as positive if more than 1 malignant epithelial cell was detected at any location on the slide; and negative if the slides were acellular or had the presence of benign cells.

On bread loafing sections histopathology, the margin was defined as positive or incomplete if neoplastic cells were present (0 mm of tumour-free margin). The margin was defined as negative if no neoplastic cells contacted with the surgical margin. Inside the negative margin category, margins were classified as narrow (0-2 mm to the surgical margin) and clean (> 2mm to the surgical margin) (Gemignani *et al.*, 2018).

3.7. Statistical analysis

Statistical analysis was performed using commercial software SPSS (IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp).

Cochran`s Q test was used to test for differences in positive margin detection rates among the 3 techniques (IC, SC and HP). McNemar test was used to test for differences in positive margin detection among intraoperative techniques (IC and SC).

Fisher exact test was calculated to evaluate association between positive margin in intraoperative techniques (IC and SC) and tumour size category (< 2cm; 2-3 cm and > 3cm) and association between positive margin in intraoperative techniques (IC and SC) and number of tumours category (1 tumour, 2-5 tumours and >5 tumours) in the 12 mastectomy specimens. Results were considered statistically significant when $p \leq 0.05$.

Frequency data from margin status analysis from the areas of interest according to the 3 assessment techniques were reported as counts and percentages.

Sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy were calculated as measures of diagnostic accuracy with their respective 95% Cis. Diagnostic accuracy was calculated for both intraoperative margin assessment techniques (IC and SC) comparing to the gold standard method (HP) in the assessment of margin status in feline mammary tumours.

4. Results

In this study, 69 margins from 12 mastectomy specimens were included. Mastectomy specimens were from 12 female cats with mean ± standard deviation body weight and age of 3.9±0.9 Kg and 12 ± 3.3 years respectively. The 12 female cats from this study included 11(91.7%) Domestic Short Hair cats and 1(8.3%) Persian cat. From these, 5 (41.7%) were intact females and 7 (58.3%) were spayed females. Regarding tumours location, 11 (91.7%) cats had bilateral presentation and 1(8.3%) unilateral. Five cats (41.7%) performed left radical mastectomy, 6 (50%) right radical mastectomy and 1 (8.3%) left partial mastectomy (as this cat underwent a partial mastectomy previously). Information summarized in Table 5 shows that cats included in the study presented different histopathological classifications. Regarding the number of tumours present in the 12-mastectomy specimen, 1 (8.3%) had 1 tumour, 4 (33.3%) had 2 to 5 tumours and 7 (58.3%) had more than 5 tumours. Mean ± standard deviation for the largest tumour size in each mastectomy specimen was 2.8± 1.8 cm. Only 4 (33%) cats presented with tumour ulceration, 2 (16.7%) cats had lymphovascular invasion and both had positive margins in intraoperative cytology (IC and/or SC). In 6 (50%) cats, lymph nodes were positive on HP. According to TMN stage, 1 (8,3%) cat was Stage I, 1(8.3%) Stage II, 7 (58.3%) Stage III and 3 (25%) Stage IV. Finally, during the study period (16 months) 3 (25%) cats were euthanized with complications related to mammary tumours.

Table 5. Sample characterization and follow-up per animal during the study period.

Patient	No. tumours	Largest tumour size	Ulceration	LI	Lymph node	Histopathology classification	TMN stage	Technique			Follow-up
								IC	SC	HP	
1	2-5	3.5 cm	+	-	+	Comedocarcinoma grade III	Stage III	-	-	-	Died
2	2-5	1.5 cm	-	-	+	Tubulopapillary Carcinoma grade II	Stage III	-	-	-	Alive
3	>5	6.5 cm	-	-	-	Tubulopapillary Carcinoma grade I	Stage III	-	-	-	Alive
4	1	1.5 cm	-	-	-	Tubulopapillary Carcinoma grade I	Stage I	-	-	-	Alive
5	>5	2.5 cm	-	-	+	Tubulopapillary Carcinoma grade II	Stage III	-	+	-	Died
6	>5	0.5 cm	+	-	+	Solid Carcinoma grade III Mammary adenosis	Stage IV	-	-	-	Alive
7	>5	5 cm	+	+	+	Solid Carcinoma grade II and III	Stage IV	+	+	-	Died
8	>5	5 cm	+	+	-	Tubulopapillary Carcinoma grade I and III Solid Carcinoma grade III	Stage III	-	+	-	Alive
9	2-5	2.5 cm	-	-	+	Tubulopapillary Carcinoma grade III	Stage IV	-	-	-	Alive
10	2-5	4.5 cm	-	-	-	Mammary Fibroadenomatosis	Stage III	-	-	-	Alive
11	>5	3 cm	-	-	+	Solid Carcinoma grade II Mammary adenosis	Stage III	-	-	-	Alive
12	>5	1.5 cm	-	-	-	Tubular Carcinoma grade II Mammary adenosis	Stage II	-	-	-	Alive

"LI"- Lymphovascular Invasion; "IC"- Imprint Cytology; "SC" - Scrape Cytology; "HP" – Histopathology; "-"- Negative Margin; "+" - Positive Margin.

Sixty-nine eligible margins were included in the study, since it was possible to collect samples for IC, SC and bread loafing sections histopathology. On bread loafing sections histopathology, margins were clean (> 2mm) in 58 (84%), narrow (0-2 mm) in 11 (16%) and incomplete in 0 (0%). In the narrow margins, 1(9%) was cranial lateral and 10 (91%) were deep margins (cranial or caudal). Only 1(9%) narrow margin is related with positive intraoperative cytology, namely SC, but 2 (50%) positive intraoperative cytology (1 IC and 1 SC) were from deep margins.

In Table 6, information shows that from the 69 evaluated margins, 3 (4.5 %) were positive on SC, 1(1.5%) was positive on IC, and no positive margins were detected on bread loafing sections histopathology. With Crochan`s Q test, positive margins detection rates did not differ between techniques (*P = 0.174*).

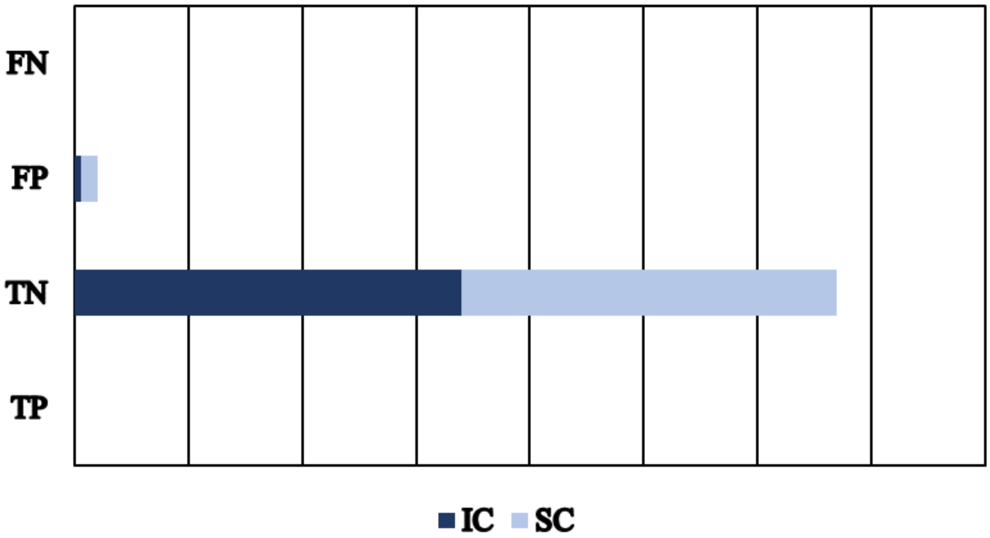
Table 6. Cross-tabulation of results of margin status analysis from the areas of interest according to the 3 assessment techniques.

Margin Assessment Technique			**Area of Interest**						**Total 69 (100)**
			Cranial 36 (52.2)			**Caudal 33 (47.8)**			
IC	SC	HP	Lateral 12 (17.4)	Medial 12 (17.4)	Deep 12 (17.4)	Lateral 11 (15.9)	Medial 11 (15.9)	Deep 11 (15.9)	
-	-	-	12 (17.4)	11 (15.9)	11 (15.9)	11 (15.9)	11 (15.9)	9 (13)	65 (94)
-	+	-	0 (0)	1 (1.5)	0 (0)	0 (0)	0 (0)	2 (3)	3 (4.5)
+	-	-	0 (0)	0 (0)	1 (1.5)	0 (0)	0 (0)	0 (0)	1 (1.5)

Data are given as number (%). "IC"- Imprint Cytology; "SC" - Scrape Cytology; "HP" – Histopathology; "-"- Negative Margin; "+" - Positive Margin.

When positive margin detection rates between intraoperative cytology techniques (IC and SC) were analysed, positive margin detection rates did not differ between techniques on McNemar test (*P= 0.625*).

Calculated Fisher exact test did not detect association between positive margin in intraoperative techniques (IC and SC) and tumour size category (*P=0.545*) and between positive margin in intraoperative techniques (IC and SC) and number of tumours category (*P= 0.427*).



	TP	TN	FP	FN
IC	0	68	1	0
SC	0	66	3	0

Figure 10. Graphical representation of the number of false negative (FN), false positive (FP), true negative (TN) and true positive (TP) from imprint cytology (IC) and scrape cytology (SC) versus histopathology (gold standard).

Regarding diagnostic accuracy for IC and SC no true positive and false negative results were detected and only true negative and false positive results were detected (Figure 10). Sensitivity and positive predictive value for IC and SC were 0%, specificity, negative predictive values and overall accuracy for IC and SC obtained high values (Table 7).

Table 7. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and overall accuracy of imprint cytology (IC), scrape cytology (SC) comparing to histopathology (HP) in the assessment of margin status in feline mammary tumours.

Margin Assessment Technique	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
IC	0	98.6 (92.2-100)	0	100 (NC)	98.6 (95.8-100)
SC	0	95.7 (87.8-99.1)	0	100 (NC)	95.7 (91-100)

Values in parentheses represent 95% CIs. "NC" - Not Calculated; "PPV" – Positive Predictive Value; "NPV" – Negative Predictive Value; "IC" - Imprint Cytology; "SC" - Scrape Cytology.

5. Discussion

This study demonstrates the feasibility of IC and SC to assess surgical margins in cats undergoing radical mastectomy, despite the large size of excised tissue.

The mean age for development of FMTs in cats is 10 to 12 years old (Morris, 2013), which is on pair with the obtained average of 12 ± 3.3 years. Unlike in dogs, obesity has not been implicated as a predisposing factor for the development of FMTs (Morris, 2013), which also correlates to the average weight of 3.9 ± 0.9 Kg of the studied patients. Unfortunately, there has been only one study that investigated the link between breed and development of FMTs (Hayes *et al.*, 1981), with all subsequent studies citing this source. They concluded that Siamese cats had twice the risk when compared to other breeds (Hayes *et al.*, 1981). In our study, 11 cats were domestic shorthair and 1 was a Persian. This does not correlate with the results obtained in Hayes *et al.*, 1981, however, the study sample in our case is very small and Hayes *et al.*, 1981 was conducted in North America more than four decades ago.

Intact cats possess a higher risk for the development of FMTs than spayed cats (Cassali *et al.*, 2020). There is a 91% reduction in the risk of development of mammary tumours in cats castrated up to 6 months old, a 86% reduction up to 1 year and a reduction of only 11% if the procedure is performed between 13 to 24 months of age (Cassali *et al.*, 2020). Unfortunately, no conclusions could be drawn in this study regarding the correlation between reproductive status and any other factor, since, in the spayed animal's group, most cases did not possess a recording of the age that the surgery occurred.

Regarding tumour number and location, 60% of cats develop multiple masses, usually ipsilateral and occasionally bilateral (Morris, 2013). The majority of cats in our study had more than 5 tumours (58.3%), with only one patient having 1 tumour (8.3%), meaning, 91.6% of patients developed multiple masses, a percentage superior than the one obtained in Morris, 2013. Interestingly, only one of our patients had unilateral FMTs.

The most common histological type is simple adenocarcinoma derived from the luminal epithelium of the mammary ducts and alveoli (Morris, 2013). Benign mammary tumours are rarely reported in cats, with the most common being fibroadenoma (Morris, 2013). Our results correlate with these statistics, only 1 tumour was benign, classified as mammary fibroadenomatosis, with all remaining tumours classified as adenocarcinomas, the most predominant being tubulopapillary carcinomas.

Prognosis is guarded for most cats with mammary tumours, with the average time between detection and death being 10 to 12 months (Morris, 2013). Table A1 assembles all prognostic factors of FMTs. Largest tumour size is correlated with prognosis: cats with the largest tumour being < 3cm have a more favourable prognosis than cats with > 3cm (Morris, 2013). Of the three deaths in our case study, only one had a largest tumour size < 3cm. The largest tumour in our case study was 6.5 cm, with the cat still being alive. However, this patient had a grade I tubulopapillary carcinoma, meaning that this was a well differentiated tumour, which is also a positive prognostic factor.

During the 16 month follow-up period, 3 cats were euthanised due to complications relating to mammary neoplasia. In these 3 cases, lymph nodes had already been invaded by cancerous cells. Although in 2 of these cases at least one cytological method detected tumoral cells, surgery had a more palliative role and it is unlike that re-excision would have augmented life span. Unfortunately, this short follow-up period does not allow conclusions to be drawn regarding the rate of local recurrence, especially in cats with negative intraoperative results, and median survival time per stage.

A histological margin free of tumour cells is considered the best predictor of improved local tumour control and is associated with improved survival and disease free intervals (Bray, 2017). However, and as previously stated, histopathology evaluates only a small fraction of the surgical margin and is frequently affected by false negatives. Even with the adoption of more suitable histological methods to the tissue at hand, such as bread loafing histopathology for mammary tissue, this risk never subsides, and the development of alternative margin assessment methods is crucial.

A feature of higher grade tumours is the migration of individual cell clusters beyond the tumour margin, being an important factor in tumour recurrence (Bray *et al.*, 2023). This is another limitation of histopathology worth mentioning, since, in this study, intraoperative positive margins stemmed from TMN stage III and IV patients and grade II or III carcinoma.

Several novel technologies for intra surgical margin assessment are in development almost exclusively in human medicine (Milovancev *et al.*, 2017; Pradipta *et al.*, 2020), with little experimentation in the veterinary world. Even in human medicine, there are elevated costs associated with these novel technologies and, therefore, poor accessibility. Cytological methods are simple and require less resources (Milovancev *et al.*, 2017), making them an accessible choice in countries and hospitals that do not have the funds to have the preferred method of intra operative margin assessment, FS. In the present study, the cytology slides were analysed in the post-operative period, with the time between collection and communication of results not quantifiable. However, in human medicine, cytology techniques are relatively fast, averaging about 10 minutes between the moment of collection to presenting the results to the surgeon, require only a pathologist to analyse the slides and pose no risk of losing diagnostic material (Bakhshandeh *et al.*, 2006; D'Halluin *et al.*, 2009). Several studies have proven these cytological methods to be equal to FS and help diminish the percentage of secondary excision in human patients (D'Halluin *et al.*, 2009; Milovancev *et al.*, 2017), making them appropriate to implement in veterinary medicine.

Due to their frequently malignant nature and their usually unfavourable prognosis, the removal of all FMTs is recommended, the best approach being two-step bilateral radical mastectomy with regional, axillary, and inguinal lymph nodes excised, even if not clinically altered (Cassali *et al.*, 2020). FMTs were chosen due to their frequently devastating prognosis and in order to uniformize results, since different tumoral origins give rise to different results, as is observed in the poor exfoliative tendencies of sarcomas (Milovancev *et al.*, 2017).

Results of the present study show a high diagnostic accuracy between the two intraoperative methods and histopathology, with an IC and SC overall accuracy of 98.6% and 95.7% respectively.

Most research in this area presents their results with PPVs, NPVs, sensitivities, and specificities, however, these statistical tests assume histological analysis as the true answer. This, of course, is incorrect. Histology relies on human analysis of the samples and human error, although minimizable, can never be excluded, leading to unavoidable false negatives, with especially catastrophic results in oncological surgery (Macios & Nowakowski, 2022). However, since most research uses these statistical tests, they were also applied in this study as a way of comparison to the results obtained in humans.

True positive results or false negative results were not observed since all 69 surgical margins analysed by bread loafing histopathology were negative, making this the reason why IC and SC sensitivity and positive predictive had a value of 0%. One false positive result was observed in IC and 3 in SC. According to D´Halluin *et al.* 2009, in human breast cancer lumpectomy, false positives in IC were related the pressing of the specimen against the glass slide and, when tumours with vascular and lymphatic emboli are pressed, the malignant cells seep out of the infiltrated ducts or vessels. Following this principle, the positive IC margin can be considered a false positive, since samples were collected by pressing the mastectomy specimen on the glass slide. However, false positives margins from SC were collected from the operative field after removing the mastectomy specimen, which suggests the possibility that these are not false positives but true positives, and failure to identify positive or incomplete surgical margins may have occurred in histopathology.

Three false positives in our study were from tumours with lymphovascular invasion detected with histopathology. According to D´Halluin *et al.* 2009, the limits of *in situ* and lobular lesions, and the extension of vascular and lymphatic emboli are poorly assessed by histopathology.

Histopathology only evaluates approximately 0.1% to 0.01% of the total circumferential surgical margin (Bray *et al.* 2023a). The pathologist must select regions of interest on macroscopic data, which is a source of sampling error (D`Halluin *et al.*, 2009). It is technically impossible to analyse the entire surface of an operative specimen, since a histological section is 5 µm thickness, and 4000 tissue sections would need to be examined in order to represent the surface of a specimen measuring 2 cm in length (D`Halluin *et al.* 2009).

In our study, true negative values were high in IC and SC methods, leading to a high specificity and a negative predictive value of 100% in the two methods.

Differences in the number of positive surgical margins, specificity and overall accuracy between IC and SC may be explained by the differences between the 2 methods in obtaining a sample. IC collected cells by apposition and SC collected cells by scraping the operating field justifying the fact that glass slides prepared with SC showed higher number of cells when compared to IC. This seems to prove the theory postulated in Kolte & Satarkar, 2010 that SC is a superior technique to IC, since, through scraping, more cells are obtained, however, the case number is too small to confidently conclude such.

When only intraoperative methods (IC and SC) were compared, despite positive margins were observed, 3 (4.5 %) in SC and 1 (1.5%) in IC, no significant differences were observed between positive margins detection rates.

Size and number of tumours present could, potentially, increase positive margin rate, the rationale behind being a more difficult surgical planning. However, Fisher's exact test did not detect associations between positive margin rate in either intraoperative method and tumour size category, and between positive margin rate in either intraoperative method and number of tumours category. Perhaps this association could occur if the surgeries are performed by less experienced surgeons. Bray *et al.*, 2023 explains that the surgeon's ability is a critical component to achieve free margins. More experienced surgeons can maintain appropriate boundaries around the entire tumour, while also remarking that for inexperienced surgeons, deeper dissections can become more challenging, with narrower excisions performed (Bray *et al.*, 2023).

None of the surgical margins evaluated by histology were incomplete, however, 91% of the narrow margins were deep margins. This highlights an essential issue to this type of surgery: while safe margins are easy to achieve in the superficial planes through large resections of tissue, the same cannot be done in the deep plane without creating large thoracic and abdominal defects in the interest of avoiding recurrences. Because complete margins are easily achieved in the superficial planes, cytological methods would be most useful in the deep planes. However, in this case, only 9% of narrow margins were associated with positive intraoperative cytology, namely SC. This is, of course, one of the aforementioned limitations of cytology. A narrow margin in histology was, in this case, described as a border of healthy tissue between 0 to 2 mm around the tumour. Cytology cannot properly assess the quantitative qualities of a margin, such that, as could happen in IC, after the glass slide is pressed against the specimen and the cells seep out of the tissue through vessels, we cannot properly know if they came from the surface of the specimen, making it an incomplete margin, or more than 2 mm from the margin, making it a complete margin. Yet, in veterinary medicine, where cosmetic result is not has a big a concern as is in BCS, these false positives would only lead to a wider resection without problematic consequences.

To the authors' knowledge, this is the first time that SC is described in veterinary medicine with the aim of classifying the status of surgical margins. SC has advantages over other intraoperative techniques that access surgical margins such as the shaved margin technique. Contrary to this technique, when SC is executed on the surgical field, it can be performed over vital structures at the edges of the resection wound bed. Due to this limitation, in a study of Milovancev *et al.* 2017, the authors did not collect samples by shaved margin technique in approximately 5% of margins. Another advantage of SC over shaved margin is related to the fact that SC has a laboratory service requirement equal to IC, which is much lower than shaved margin, since this technique requires the availability of a pathology service to prepare and read frozen sections (Milovancev *et al.*,2017). SC is less time-consuming, inexpensive, and lacks freezing artefacts when compared to shaved margin technique (Gupta *et al.*, 2019).

There were several limitations of this work related to sample size, differences between intraoperative cytology techniques (IC and SC) and histopathology, since cytology techniques have a lack of contextual tissue architecture when compared to histopathology (Milovancev *et al.* 2017). Another limitation of our work is related to the lack of inking of the critical tissue margins, since identification was obtained with margin tissue sutures. Inking of critical tissue margins by the surgeon is recommended by most pathologists, as it prevents false positive and false negative margin status classifications related to tissue distortion during fixation (Bray *et al.*, 2023). Finally, the different TMN stages presented in this study may have influenced the results and, possibly in a larger sample, grouping samples by tumor grade or TMN stage might yield different positive margin rates for the techniques.

6. Conclusion

The results obtained agreed with previous studies. IC and SC were easy to perform techniques and, although not applied in the present study, the whole process was fast and the results could have been communicated to the surgeon in under 10 minutes. These techniques are quite researched in human medicine, with only one study published in veterinary medicine (Milovancev *et al.*, 2017). Bray *et al.*, 2023 defends that their research in veterinary medicine is crucial in increasing the life span and quality of life in the veterinary patient. Furthermore, and as previously stated, histopathology possesses several limitations that inhibit a reliable clean margin diagnosis to be obtained.

These techniques could be more relevant in the assessment of adequacy of the deep plane margins, since this seems to be the location where the majority of incomplete or narrow margins will occur. Since both these cytological methods and histopathology present their flaws, it would be wiser to use them as complements to each other rather than alone.

Further studies with larger number of animals are needed to understand the clinical relevance of IC and SC in feline mammary tumours prognosis as complementary techniques to histopathology.

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8. Annexes

Table A1. Prognostic factors for FMTs (Adapted from Morris, 2013)

Factor	Details
Tumour size	< 3cm – median survival of 21-24 months > 3cm – median survival of 4-12 months
Clinical Stage	Stage I – median survival 29 months Stage II – median survival 12.5 months Stage III – median survival 9 months Stage IV – median survival 1 month
Surgical extent	Radical surgery (mammary strip) reduces recurrence rate compared with more conservative mastectomy
Histopathological grade	Well differentiated – 100% survival at 1 year after surgery Poorly differentiated – 0% survival at 1 year after surgery
Mitotic index	<2 mitotic figures per high power field give longer survival

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IMPLEMENTATION OF TWO INTRAOPERATIVE CYTOLOGICAL TECHNIQUES FOR EVALUATION OF CLEAN STATUS MARGINS IN FELINE MASTECTOMY

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Introduction

The main goal of oncological surgery is the removal of all neoplastic cells and achieve a clean margin status to prevent local recurrence and prolong patient's life. To improve patient care in feline mammary tumours surgery, it is necessary to develop or improve techniques that improve recognition of residual tumour cells during the intraoperative period and within the surgical field. Imprint cytology and scrape cytology during the intraoperative period, provide a reasonable assessment of the adequacy of surgical resection limits, helping the surgeon make an intra-operative decision regarding proceeding with further extension of surgical margins.

This study aims to determine the agreement between margin status with imprint cytology (IC) and scrape cytology (SC) in unilateral radical mastectomy for feline mammary tumours surgical treatment.

Materials and Methods

A total of 12 female cats were submitted for unilateral radical mastectomy. Intraoperative surgical field margins assessment was accomplished through SC and fresh mastectomy specimen margins assessment by IC. A total of 69 cranial and caudal margins (lateral, medial and deep) were evaluated by the 2 methods (Fig.1). Agreement between methods were evaluated by the Cohen Kappa Coefficients. McNemar test was used to test for differences in positive margin detection rates between methods.

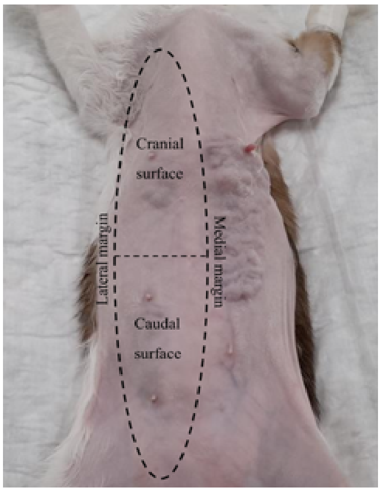

Figure 1. Illustration of mastectomy divisions in order to perform SC, IC.

Results

Positive margins detection rates and agreement between intraoperative cytology methods (IC and SC) were analysed (Table 1.). Positive margins detection rates did not differ between methods on McNemar test (*P= 0.625*) and a moderate agreement between the calculated Cohen Kappa coefficient (0.489).

Table 1. Cross-tabulation of results of margin status analysis from the areas of interest according to the 2 assessment techniques.

Margin Assessment Method		Area of interest: Cranial (n=36)			Caudal (n=33)			Total (n= 69)
IC	SC	Lateral (n=12)	Medial (n=12)	Deep (n=12)	Lateral (n=11)	Medial (n=11)	Deep (n=11)	
-	-	12 (100%)	11 (91.7%)	11 (91.7%)	11 (100%)	11 (100%)	9 (81.8%)	65 (94.2%)
-	+	0 (0%)	1 (8.3%)	0 (0%)	0 (0%)	0 (0%)	2 (18.2%)	3 (4.3%)
+	-	0 (0%)	0 (0%)	1 (8.3%)	0 (0%)	0 (0%)	0 (0%)	1 (1.5%)

Conclusions

IC and SC proved to be reliable methods in the assessment of clean status margins during the intraoperative period. SC was found to have a higher positive margin detection rate in feline mammary tumours when compared to IC, but no significant differences were observed between methods.

IC and SC are feasible techniques capable of being used during the intraoperative period in veterinary medicine, since they rely only on the availability of a pathologist and the results can be communicated in less than 10 minutes to the surgeon.

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